



Saturated fatty acid-induced miR-195 impairs insulin signaling and glycogen metabolism in HepG2 cells



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ABSTRACT

MicroRNAs (miRNAs) play an important role in insulin signaling and insulin secretion, but the role of miRNAs in the association between obesity and hepatic insulin resistance is largely unknown. This study reports that saturated fatty acid (SFA) and high fat diet (HFD) significantly induce miR-195 expression in hepatocytes, and that the insulin receptor (INSR), not insulin receptor substrate-1 (IRS-1), is a direct target of miR-195. Furthermore, the ectopic expression of miR-195 suppresses the expression of INSR, thereby impairing the insulin signaling cascade and glycogen synthesis in HepG2 cells. These findings suggest that the dysregulation of miR-195 by SFA is a detrimental factor for hepatic insulin sensitivity.

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1. Introduction

Obesity is a rapidly increasing global health problem that is closely linked to the pathogenesis of insulin resistance [1]. Insulin resistance is defined as an inadequate response of the peripheral tissues to insulin, and is a hallmark of type 2 diabetes mellitus (T2DM) and metabolic syndrome [2,3]. The ectopic accumulation of fatty acids in the liver, termed hepatic steatosis, results in a dysregulation of the cellular metabolism and can specifically provoke insulin resistance in hepatocytes [1,4]. Several lines of evidence have been suggested to elucidate the implications of excess saturated fatty acids (SFA) on hepatic insulin resistance, such as the accumulation of diacylglycerol (DAG) and ceramide, oxidative stress, mitochondrial dysfunction, etc. [4,5]. Despite these findings, the precise mechanisms through which elevated SFA results in hepatic insulin resistance are unclear.

The liver plays a critical role for carbohydrate homeostasis that regulates the balance between the glycogen metabolism and

gluconeogenesis, according to the metabolic demands [2,4]. In this process, insulin signaling is one of the most important regulatory mechanisms, which is initiated by insulin binding to insulin receptor (INSR). This ligand-receptor interaction induces tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), subsequently transmitting the signals from IRS-1 to down-stream enzymes, such as PI3K, PDK-1 and Akt2 [6]. On the other hand, an overload of SFA, such as palmitate, increases the intermediate lipid metabolites including ceramide and DAG, which leads to insulin resistance resulting from the serine phosphorylation of IRS-1 via protein kinase C, JNK, and IKK [1,7]. Although inappropriate post-translational modification and down-regulation of IRS-1 by SFA have detrimental effects on insulin sensitivity, several studies have suggested that the down-regulation of INSR also contributes to the pathogenesis of insulin resistance and T2DM [8–11]. Mice lacking the INSR gene suffer from the rapid onset of hyperglycemia and hyperinsulinemia, followed by diabetic ketoacidosis and early postnatal death [12]. The conditional knockouts of INSR in the liver result in the development of hyperglycemia, hyperlipidemia, hyperinsulinemia, and obesity [13]. A large number of studies already have revealed a modest decrease in INSR in T2DM patients [10,11]. These findings support the central role of hepatic INSR in the whole body insulin sensitivity. On the other hand, the

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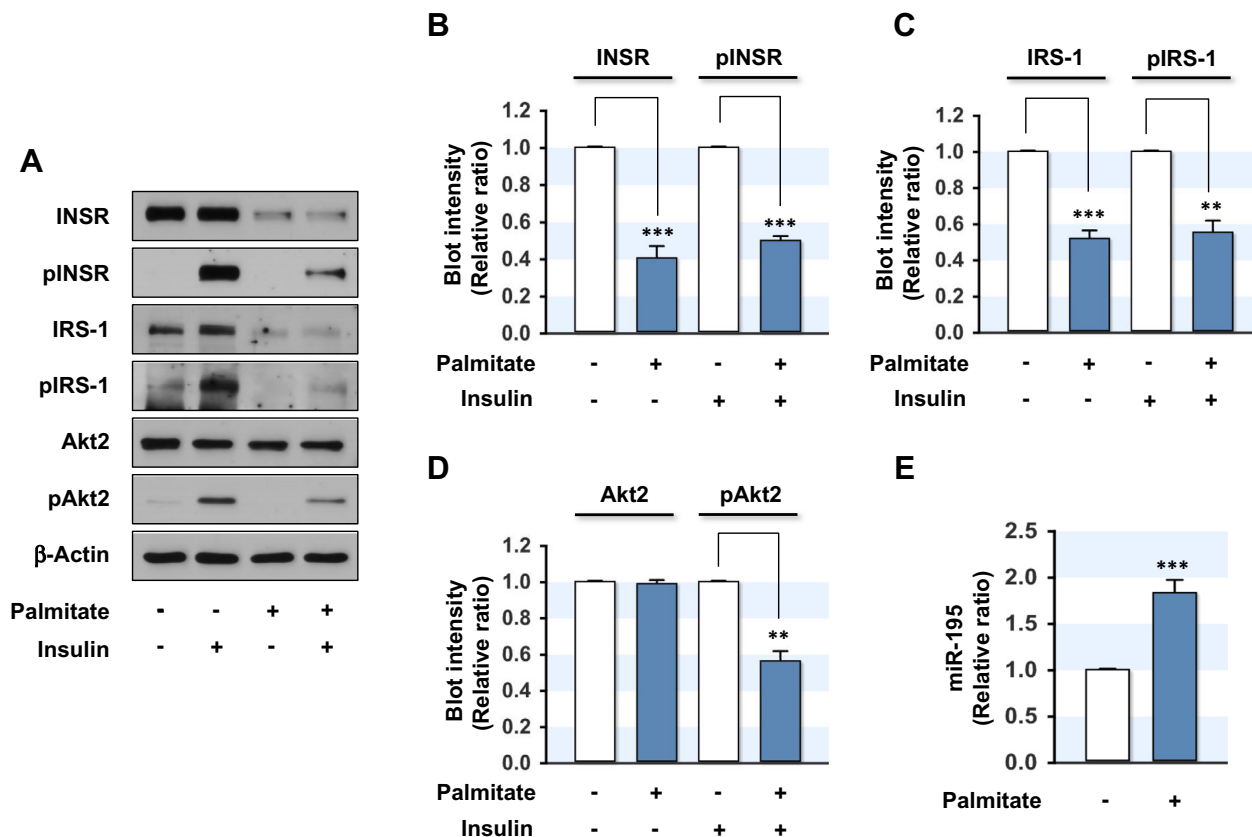


Fig. 1. SFA palmitate-induced insulin resistance and up-regulation of miR-195 in HepG2 cells. (A) HepG2 cells were preincubated with or without palmitate (0.5 mM) for 18 h. The cells were serum-starved for 4 h and then, incubated in the presence or absence of insulin (100 nM) for 30 min. The expression (INSR, IRS-1 and Akt2) and phosphorylation (pINSR, pIRS-1 and pAkt2) of insulin signaling intermediates were analyzed by immunoblot. The immunoblot is representative of five independent experiments. (B–D) The immunoblot intensities were quantified by densitometry, and were normalized to the amount of β -actin. Blot intensities are expressed as the relative ratio, where the intensity of the control (open column) was set to one. (E) The expression of miR-195 was quantified by qRT-PCR from control and palmitate-treated (0.5 mM, 18 h) HepG2 cells. The values are expressed as the relative ratio, where the intensity of the control (open column) was set to one. Values are means \pm S.E.M. from at least three independent experiments; ** P < 0.01; *** P < 0.001.

mechanism underlying the SFA or obesity-induced down-regulation of INSR is largely unknown.

MicroRNAs (miRNAs) are a family of 19–25-nucleotide, highly conserved, endogenous non-coding RNAs that modulate target gene expression at the post-transcriptional level [14]. Their biological function is known to suppress gene expression mainly by base-pairing to the 3' untranslated regions (3'UTRs) of the target mRNAs, eventually triggering either degradation of the target mRNAs or the suppression of translation [14]. A growing body of evidence suggests that miRNAs regulate a range of gene functions in both normal and pathological states, and the aberrant expression of miRNAs are involved in the development of many diseases, such as proliferative, neurodegenerative and metabolic diseases [14,15]. Since the first discovery that miRNA is actively involved in metabolic regulation, such as amino acid catabolism [16], a number of studies have suggested that miRNAs play significant roles in both glucose homeostasis and lipid metabolism implicated in insulin resistance and T2DM [17,18]. Previous studies have shown that the insulin resistance induced by a high fat diet (HFD) up-regulates certain miRNAs, such as miR-96, miR-126, and miR-195, in the liver, and that miR-96 and miR-126 impair hepatic insulin signaling and the glucose metabolism by suppressing IRS-1 expression [18,19]. Although the aberrant expression of miR-195 is involved in a range of diseases, such as cancer, cardiac hypertrophy and heart failure [20], the implication of miR-195 in hepatic insulin sensitivity is lacking. The present study found that

SFA and HFD up-regulates miR-195 expression in hepatocytes. In addition, this study showed, for the first time, that miR-195 suppresses the expression of INSR via an direct binding site in its 3'UTR, causing impaired insulin signaling and glycogen metabolism. Therefore, miR-195 is causally linked to the development of insulin resistance by SFA.

2. Materials and methods

2.1. Cell culture, palmitate treatment, and transfection of miRNA mimics

HepG2 cells (ATCC, #77400) derived from human liver carcinoma were cultured in MEM alpha containing 10% FBS and 1% penicillin–streptomycin. For palmitate-induced insulin resistance, the cells were treated with BSA-conjugated palmitate (0.5 mM) for 18 h [21]. For insulin stimulation, the cells were serum-starved for 4 h, and then stimulated with insulin (100 nM, Sigma) for 30 min [22]. For miRNA mimics or plasmids transfection, HepG2 cells were reverse-transfected using G-fectin (Genolution, Korea) according to the manufacturer's instructions.

2.2. Animal and HFD-induced insulin resistance

All animal procedures were performed in accordance with the rules of the Animal Use and Care Committee at Dongguk

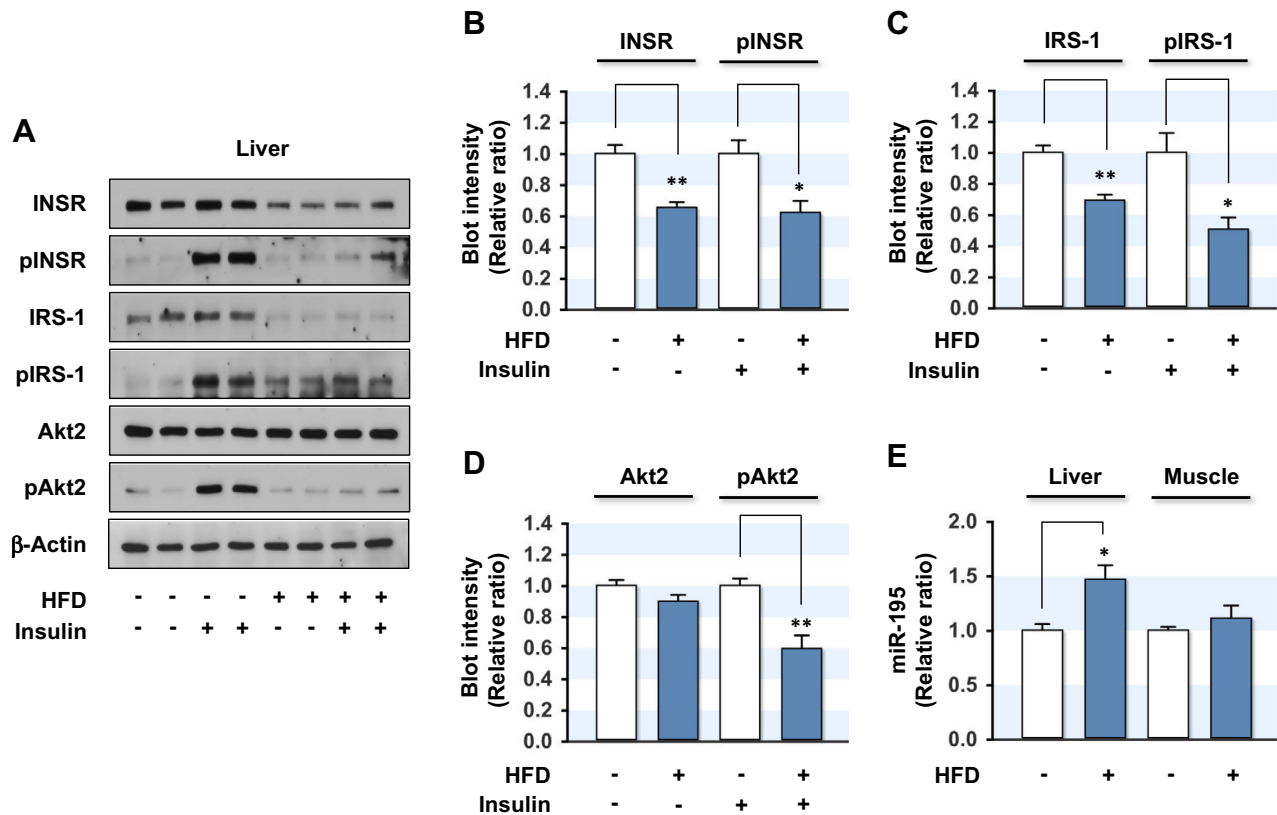


Fig. 2. HFD-induced insulin resistance and up-regulation of miR-195 in DIO mice. Mice were fed either NFD or HFD for 14 weeks. HFD developed impaired glucose tolerance and hyperglycemia, as shown in *Suppl. 2*. (A–D) Mice fed NFD (open column) and HFD (closed column) were fasted overnight and sacrificed 20 min after i.p. injections of vehicle or insulin (2U/kg body weight). Representative immunoblots (2 from 10 mice/group) obtained from liver lysates were shown in A. The expression (INSR, IRS-1 and Akt2) and phosphorylation (pINSR, pIRS-1 and pAkt2) of insulin signaling intermediates in the liver were analyzed by densitometry, and were normalized to the amount of β -actin. The values are expressed as the relative ratio, where the intensity of the control (open column) was set to one. (E) The expression of miR-195 were quantified by qRT-PCR from mice fed NFD (open column) and HFD (closed column). The values are expressed as the relative ratio, where the intensity of NFD mice (open column) was set to one. Values are means \pm S.E.M. from at least six individuals; * $P < 0.05$; ** $P < 0.01$.

University. C57BL/6 mice were purchased from Central Lab. Animal, Inc (Seoul, Korea). The mice were kept in a temperature-controlled facility with a 12:12 h light–dark cycle and given access to food and water ad libitum. At 6 weeks of age, the mice were fed either a normal fat diet (NFD, 11% calories from fat) or a HFD (60% calories from fat; Dyets Inc., Bethlehem, PA) for 14 weeks. The body weight was measured every week. At the end of experiment, the mice were fasted overnight and subjected to an oral glucose tolerance test (OGTT) and biochemical analysis, as described previously [21].

2.3. RNA preparation and quantitative real-time RT-PCR (qRT-PCR)

The total cellular RNAs were isolated using a miRNeasy Mini Kit (Qiagen). For qRT-PCR, cDNA was synthesized using M-MLV reverse transcriptase (Promega) and amplified with oligo dT (Bionics, Seoul, Korea), as described elsewhere [19]. To measure the expression levels of miRNAs, the cDNAs were synthesized using a miScript reverse transcription Kit (Qiagen) and subjected to qRT-PCR using a miScript SYBR Green PCR Kit (Qiagen) in Light-CyclerTM480 (Roche-Applied Science, Mannheim, Germany). The U6 snRNAs was used as the internal control.

2.4. Dual luciferase reporter assay

To generate the reporter gene constructs, human INSR (387nt) or IRS-1 (110nt) 3'UTR containing either the wild-type miR-195 binding sites (INSR 3Uwt or IRS-1 3Uwt) or the mutated miR-195 binding site (INSR 3Umut) was cloned into the pmirGLO Dual-

luciferase miRNA target expression vector (Promega). The luciferase assays were performed using a Dual luciferase reporter system (Promega) according to the manufacturer's instructions. Briefly, L6 GLUT4myc cells were cotransfected in 12-well plates with miR-195 mimic or scrambled control miRNA mimic (scRNA) and pmirGLO vectors containing the empty, wild-type or mutant 3'UTRs. After 24 h, the cells were lysed using a passive lysis buffer. The Firefly and Renilla luciferase activities were measured on a Sirius L (Titertek-Berthold, Germany). The relative luciferase activity was calculated by normalizing the ratio of Firefly/Renilla luciferase to that of the scRNA-transfected cells.

2.5. Glycogen assay

HepG2 cells were reverse-transfected with miR-195 mimic or scRNA. After 48 h, the cell culture medium was changed to MEM alpha without FBS for 4 h, and then incubated with DMEM containing high glucose levels in the absence or presence of insulin (100 nM) for 90 min. After washing three times with ice-cold PBS, the glycogen contents of the cells were measured using a Glycogen colorimetry assay Kit II (Biovision) according to the manufacturer's instructions.

2.6. Antibodies and immunoblotting

The antibody against IRS-1 was purchased from Upstate (Lake Placid, NY). The antibody against phospho-IRS-1 and β -actin was supplied by Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were obtained from Cell Signaling Technology (Beverly,

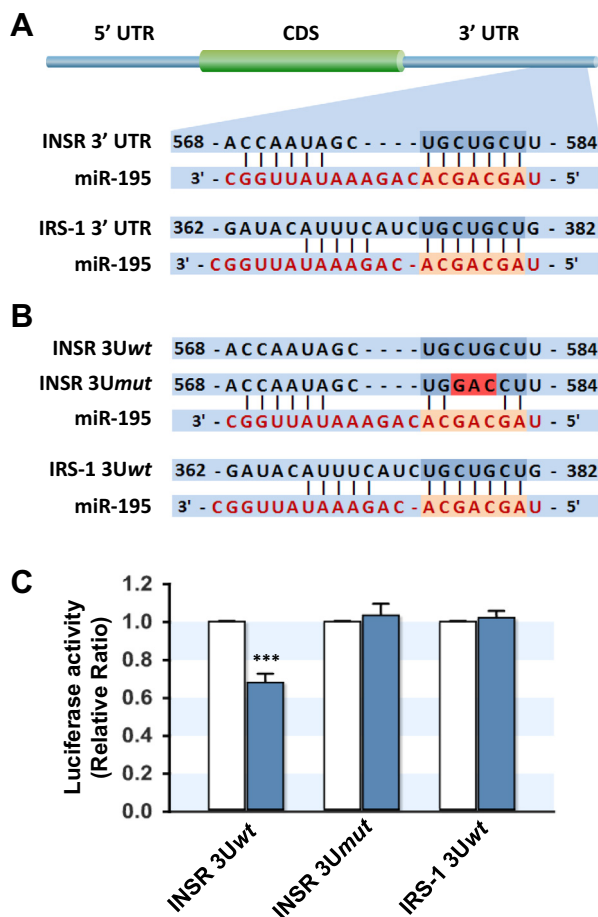


Fig. 3. Targeting sites of miR-195 in the 3'UTRs of *INSR* and *IRS-1*, and an assessment of its binding by the Dual luciferase reporter assay. (A) The seed sequence of miR-195 was predicted to target *INSR* and *IRS-1* 3'UTRs. (B) For the Dual luciferase reporter assay, 3'UTRs of the *INSR* and *IRS-1* genes were inserted downstream of a firefly luciferase open reading frame (wild-type: INSR 3Uwt and IRS-1 3Uwt). The mutated 3'UTR of the *INSR* gene lacking the miR-195 binding sites (INSR 3Umut) were examined. (C) INSR 3Uwt, INSR 3Umut, or IRS-1 3Uwt construct was cotransfected with either scRNA control (open column) or designated miR-195 mimic (closed column) into L6 GLUT4myc cells, as described in the Section 2. The relative luciferase activities were plotted against that of the 3Uwt cotransfected with scRNA control (open column), which was set as one. The values are expressed as the means \pm S.E.M. from three independent experiments. *** $P < 0.001$.

MA). Immunoblotting analysis was performed as described elsewhere [19]. The proteins were visualized using ECL Western Blotting Detection Reagents (GE Healthcare, UK). The immunoblot intensities were quantified by densitometry using an analytical scanning system (Alpha Imager HP, Alpha Innotech).

2.7. Database and statistical analysis

The targeting sites on *INSR* or *IRS-1* 3'UTR of miRNAs were analyzed using a TargetScan (<http://www.targetscan.org/index.html>) and PicTar (<http://www.pictar.org>). The values are expressed as the means \pm S.E.M. from at least three independent experiments. Where applicable, the significance of the difference was analyzed using a Student's *t* test for unpaired data.

3. Results

3.1. SFA impairs insulin signaling and induces miR-195 expression

To examine the effect of SFA on insulin signaling in hepatocytes, HepG2 cells were treated with palmitate (0.5 mM for 18 h), which

is the most abundant SFA in the blood. Palmitate treatment reduced the protein expression of INSR and IRS-1 in HepG2 cells considerably (Fig. 1A–C) compared to the vehicle-treated control, whereas the expression of Akt2 and β -actin were unaffected (Fig. 1A and D). Moreover, palmitate inhibited significantly the insulin-stimulated phosphorylation of INSR concomitant with the insulin signaling intermediates, such as IRS-1 and its downstream molecule, Akt2 in HepG2 cells (Fig. 1A–D). These results show that SFA palmitate impairs insulin signaling in HepG2 cells by reducing INSR and IRS-1 expression. According to previous reports, the expression of miR-195 is increased significantly in the liver of a T2DM animal [23] and in the plasma from T2DM patients [24]. As palmitate reduced the expression of INSR and IRS-1 in HepG2 cells, the expression of miR-195, which presumably target both *INSR* and *IRS-1* 3'UTR, was next analyzed. Consistent with previous studies, palmitate increased the expression of miR-195 significantly in HepG2 cells (Fig. 1E) in a dose- and time-dependent manner (Suppl. 1A–D), suggesting that miR-195 may be involved in the reduction of INSR and IRS-1, as well as in impaired insulin signaling by SFA.

3.2. Expression of miR-195 is increased in the liver of diet-induced obesity (DIO) mice

DIO was reported to impair insulin signaling in hepatocytes and myocytes [1]. Therefore, we examined how HFD affects the expression of miR-195 and how this contributes to the development of insulin resistance. In the present study, HFD (60% kcal from fat for 14 weeks) in mice increased the body weight, fasting blood glucose significantly (Suppl. 2A), and impaired the whole-body disposal of blood glucose (Suppl. 2B) compared to the normal fat diet (NFD), clearly showing that DIO mice developed hyperglycemia and insulin resistance. Moreover, the protein expression levels of INSR and IRS-1 were reduced drastically in the liver of DIO mice, whereas Akt2 and β -actin were unaffected (Fig. 2A–D). The insulin-stimulated phosphorylation of INSR and insulin signaling intermediates was also analyzed in the liver of DIO mice. As expected, HFD reduced significantly the insulin-stimulated phosphorylation of INSR, IRS-1, and Akt2, resulting mainly from the reduced expression of INSR and IRS-1 (Fig. 2A–D). The miR-195 level in insulin-responsive tissues, such as liver and skeletal muscle, from DIO mice was next analyzed. Interestingly, the miR-195 level was up-regulated significantly in the liver of DIO mice, but not in the skeletal muscle (Fig. 2E). These findings suggest that HFD increases the miR-195 expression in hepatocytes with a concomitant decrease in INSR and IRS-1 protein expression, suggesting that miR-195 may participate in the development of hepatic insulin resistance in DIO mice.

3.3. INSR gene is a direct target of miR-195

Based on *in silico* analysis using TargetScan and PicTar, the 3'UTRs of the *INSR* and *IRS-1* genes contain the predicted conserved binding sites for miR-195. Because miR-195 expression in hepatocytes was up-regulated by SFA and HFD, an increase in miR-195 may be involved in the reduction of INSR and IRS-1 by SFA. Therefore, Dual luciferase reporter assay was performed to determine if miR-195 promotes INSR and/or IRS-1 down-regulation by directly targeting their 3'UTRs. Alignment analysis showed that human *INSR* 3'UTR contains a putative conserved binding site for miR-195 (Fig. 3A). Luciferase constructs containing a putative binding site of miR-195 identified in *INSR* 3'UTR (wild-type; INSR 3Uwt), or mutated nucleotides of the same (INSR 3Umut) in pmirGLO vector were developed (Fig. 3B). Reporter constructs, which included a firefly luciferase cassette to allow normalization of the internal Renilla luciferase activity, were cotransfected transiently together

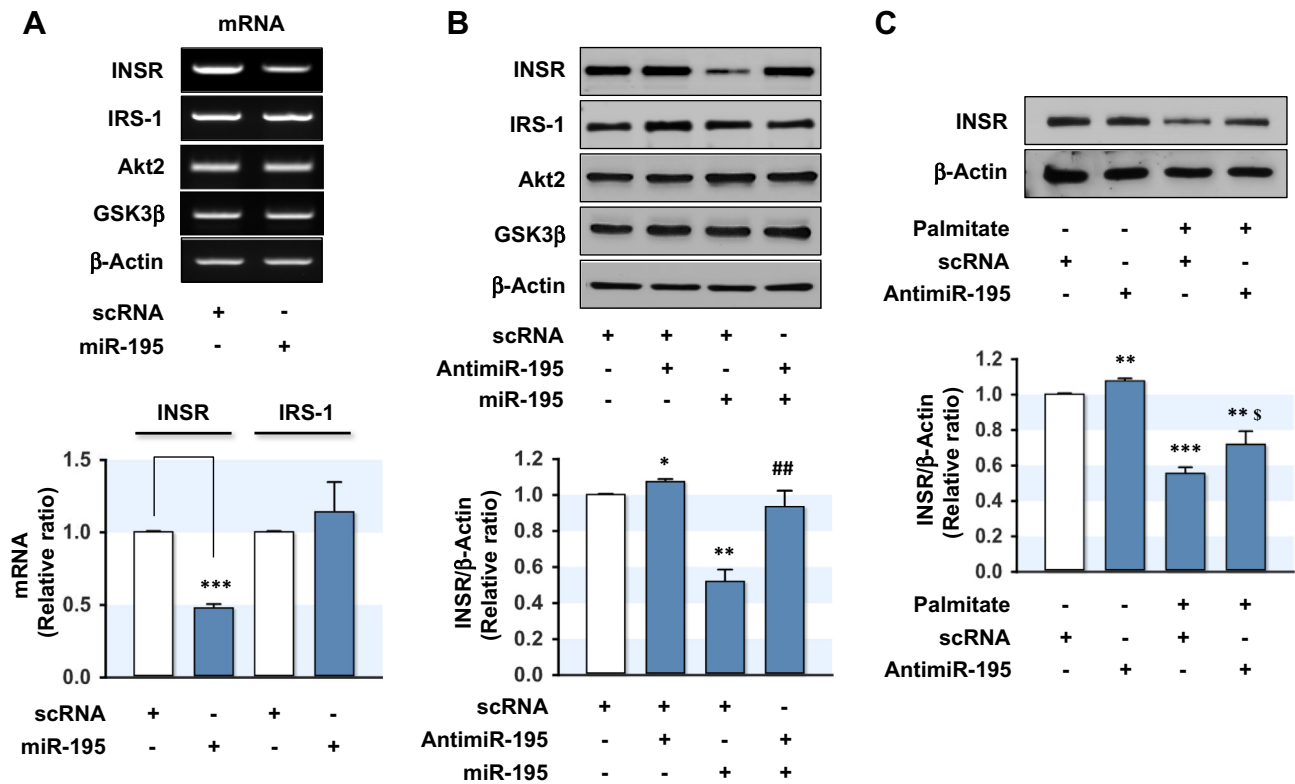


Fig. 4. Effect of miR-195 on the expression of INSR and IRS-1. (A) HepG2 cells were transfected with 200 nM of scRNA control or miR-195 mimic. The mRNA level was analyzed after 24 h of transfection by RT-PCR (upper panel), and the mRNAs of INSR and IRS-1 were quantified by qRT-PCR from scRNA control (open column) and miR-195 mimic (closed column)-transfected cells (lower panel). (B) HepG2 cells were cotransfected with scRNA (100 nM), AntimiR-195 (100 nM) and/or miR-195 mimic (100 nM), and the expression of insulin signaling intermediates was analyzed after 48 h transfection by immunoblotting. (C) The cells were transfected with 200 nM of scRNA or AntimiR-195, and additionally incubated in the presence or absence of palmitate (0.5 mM for 18 h) after 30 h of transfection. The immunoblot intensities of INSR/ β -actin were quantified by densitometry, and are expressed as the relative ratio where the intensity of the scRNA control was set to one (open column). Values are means \pm S.E.M. from at least four independent experiments; * P < 0.05; ** P < 0.01; *** P < 0.001 vs scRNA control; ## P < 0.01 scRNA + miR-195 vs AntimiR-195 + miR-195; § P < 0.05; palmitate + scRNA vs palmitate + AntimiR-195.

with miR-195 mimic or scRNA, as described in the Method. The transfection of miR-195 mimic in the presence of the INSR 3Uwt construct resulted in a significant decrease in luciferase activity compared to its mutant counterpart INSR 3Umut (Fig. 3C). This shows that miR-195 targets INSR 3'UTR directly and represses INSR expression at the translational level. Next, a highly conserved putative binding region within the 3'UTR of human IRS-1 gene for miR-195 (Fig. 3A) was cloned into the pmirGLO vector (wild-type; IRS-1 3Uwt) (Fig. 3B), and subjected to Dual luciferase reporter assay. The luciferase activity of IRS-1 3Uwt was not reduced significantly by miR-195 mimic, compared to scRNA control (Fig. 3C). This suggests that miR-195 does not target IRS-1 3'UTR directly and is not involved in the repression of IRS-1 expression at the translational level.

3.4. Expression of INSR is repressed by miR-195 at the post-transcriptional level

Because INSR was revealed as a direct target of miR-195, the ectopic expression of miR-195 should reduce the expression of INSR, leading to insulin resistance in hepatocytes. To examine this further, HepG2 cells were reverse-transfected with miR-195 mimic using G-fectin. Measurement of the transfected miRNA by qRT-PCR indicated that miR-195 was increased by >2000-fold, to a level vastly greater than the endogenous miR-195 (Suppl. 3). Therefore, all miRNAs were transfected at the concentration of 200 nM with same transfection condition, and then, the expressions of mRNA and protein levels of INSR and insulin signaling intermediates were analyzed. The ectopic expression of miR-195 showed a significant

decrease in INSR mRNA, concomitant with the substantial suppression in INSR protein, compared to scRNA control (Fig. 4A and B). On the other hand, the ectopic expression of miR-195 did not affect the mRNA and protein levels of insulin signaling intermediates, such as IRS-1, Akt2 and GSK3 β in HepG2 cells (Fig. 4A and B). Moreover, the transfection of 2'-O-methyl-modified antisense oligonucleotide against miR-195 (antimiR-195) in hepatocytes increased INSR expression significantly compared with scRNA control, and completely abolished miR-195-induced INSR suppression (Fig. 4B). These results suggest that miR-195 down-regulates the mRNA and protein expression of INSR without apparently changing IRS-1 expression in HepG2 cells. To further confirm INSR regulation by miR-195 in palmitate-treated HepG2 cells, antimiR-195 was transfected and incubated in the presence or absence of palmitate (Fig. 4C). AntimiR-195 partially but significantly rescued palmitate-induced INSR suppression in HepG2 cells, indicating that up-regulation of miR-195 contributes to palmitate-induced INSR suppression in hepatocytes.

3.5. Ectopic expression of miR-195 impairs insulin signaling and glycogen metabolism

As miR-195 reduces the mRNA and protein of INSR, the ectopic expression of miR-195 might be causally linked to impaired insulin signaling and glycogen metabolism in HepG2 cells. To determine if miR-195 is involved in the development of insulin resistance, we analyzed the insulin-stimulated phosphorylation of insulin signaling intermediates and glycogen synthesis in the presence or absence of the ectopic expression of miR-195 in HepG2 cells.

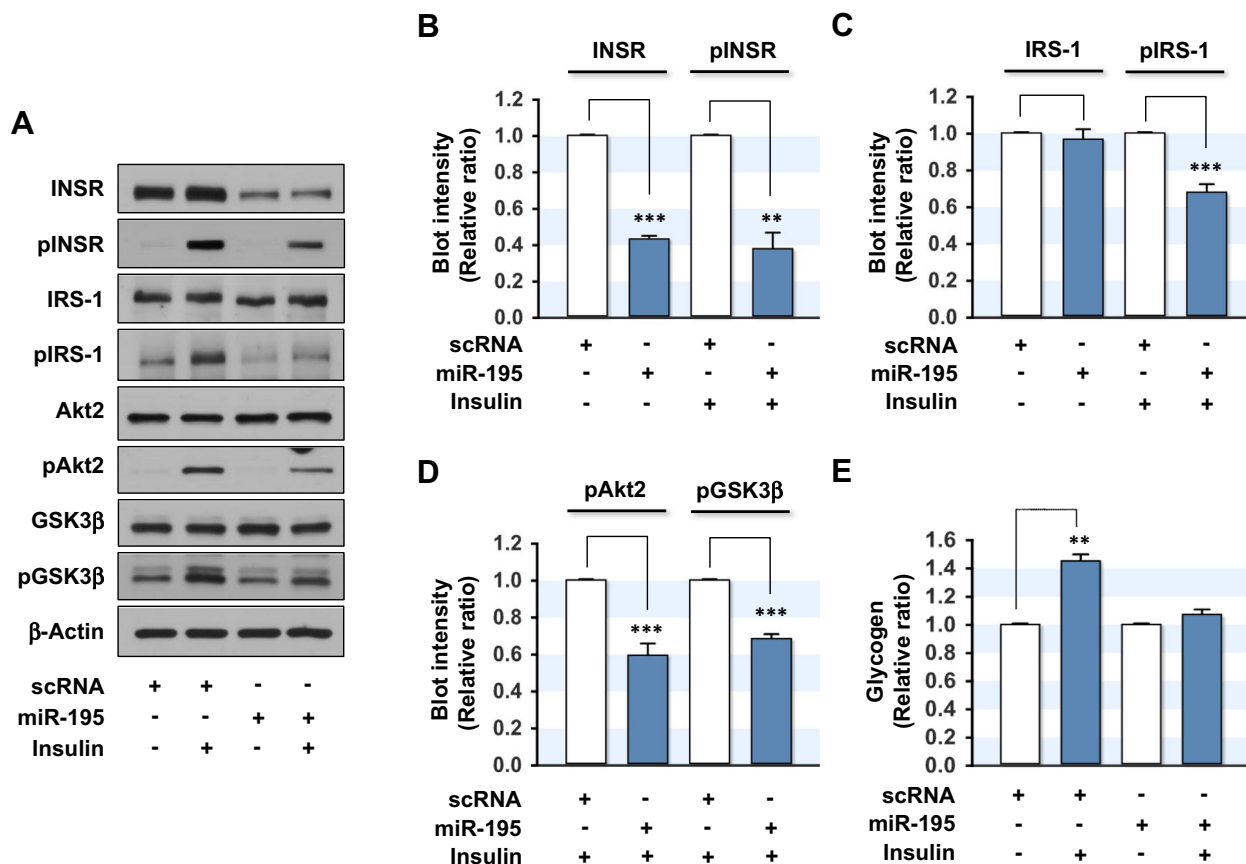


Fig. 5. Impaired insulin signaling and insulin-stimulated glycogen synthesis by the ectopic expression of miR-195. HepG2 cells were transfected with scRNA control or miR-195 mimic. After 48 h of transfection, the cells were incubated in the presence or absence of insulin (100 nM) for 30 min, and were subjected to immunoblotting. (A) The protein expression (INSR, IRS-1, Akt2, and GSK3β) and phosphorylation (pINSR, pIRS-1, pAkt2, and pGSK3β) levels of the insulin signaling intermediates were analyzed by immunoblotting. The immunoblot is representative of five independent experiments. (B–D) The immunoblot intensities were quantified by densitometry, and were normalized to the amount of β-actin. Blot intensities are expressed as the relative ratio, where the intensity of the control (open column) was set to one. (E) The glycogen contents were quantified in the presence (closed column) or absence (open column) of insulin (100 nM) from scRNA control and miR-195 mimic-transfected HepG2 cells. The values are expressed as the relative ratio, where the intensity of the normal control was set to one. Values are means ± S.E.M. from at least four independent experiments; ****P* < 0.001.

The expression of INSR was reduced significantly (~60%) by the ectopic expression of miR-195, whereas the expression of IRS-1, Akt2 and GSK3β were unaffected compared to scRNA control (Fig. 5A–C). The ectopic expression of miR-195 also reduced significantly the insulin-stimulated phosphorylation of INSR and its downstream molecules, IRS-1, Akt2 and GSK3β, in HepG2 cells (Fig. 5A–D). This was attributed mainly to the reduced expression of INSR. In addition, this study examined how miR-195 affects the insulin-stimulated glycogen synthesis in the presence or absence of insulin (Fig. 5E). In the control, insulin increased the cellular glycogen content significantly, whereas insulin-stimulated glycogenesis was barely observed after the ectopic expression of miR-195 in HepG2 cells. Therefore, the ectopic expression of miR-195 impairs insulin signaling and insulin-stimulated glycogen metabolism in HepG2 cells through the repression of INSR expression.

4. Discussion

The key findings of this study were that miR-195 is up-regulated by SFA, leading to impaired insulin signaling and glycogen metabolism through the suppression of INSR in HepG2 cells. The cellular treatment of SFA palmitate resulted in a significant increase in miR-195 (Fig. 1), and miR-195 decreased INSR expression directly through post-transcriptional suppression (Figs. 3 and 4), resulting in insulin resistance in HepG2 cells (Fig. 5). Moreover, miR-195 was up-regulated in the liver of DIO mice (Fig. 2). There-

fore, these findings reveal a novel mechanism whereby the induction of miR-195 by SFA is involved in the development of hepatic insulin resistance.

Hsa-miR-195 is an intronic miRNA of a hypothetical protein, LOC284112, which is located on chromosome, 17p13.1 [25]. Based on the expression profiling and target validation studies by ectopic expression, the aberrant expression of miR-195 has been reported to be associated with multiple diseases, such as cancer, cardiac hypertrophy, and heart failure [20]. Recently, miR-195 was reported to be the first miRNA that targets GLUT3, and is involved in the glucose metabolism of bladder cancer cells [26]. In the present study, the treatment of SFA palmitate up-regulated the expression of miR-195 in HepG2 cells. Similarly, the expression of miR-195 was also induced by palmitate in cardiomyocytes, leading to apoptosis through the down-regulation of Sirt1 and Bcl-2 [27]. Interestingly, we also found that the expression of miR-195 was increased significantly in the liver of the DIO mice, suggesting that the up-regulation of miR-195 may be actively involved in the development of insulin resistance and T2DM. These findings are consistent with previous studies. Based on global miRNA expression analysis, the expression of miR-195 is increased significantly in the liver of a Goto-Kakizaki rat, a T2DM animal model [23]. The miR-195 level is significantly increased in the retina of diabetic rats, leading to diabetic retinopathy complications [28]. Furthermore, Karolina, *et al.* recently found that the miR-195 level in plasma is correlated positively with the body mass index (BMI)

and blood pressure [29], and cross-sectional studies revealed a drastic increase in the miR-195 level in the plasma from T2DM patients [24]. Therefore, miR-195 might be one of the components in SFA-induced lipotoxicity in the cells. Hence, the induction of miR-195 in the liver appears to be a risk factor for insulin resistance and T2DM. Moreover, miR-195 may be used as a biomarker for diagnostic and therapeutic purposes because it is readily detectable in the circulatory blood. Further study is clearly warranted.

To the best of the authors' knowledge, there is no evidence demonstrating the mechanism through which excess SFA up-regulates the expression of miR-195. *In silico* analysis, however, suggests that several transcription factors, such as SREBPs, PPARs, CEBPA, and p53, may bind to specific sites on the miR-195 gene promoter regions. Because the activation of SREBPs are known to be essential for diabetic hepatic steatosis and carbohydrate-induced hypertriglyceridemia [30], the miR-195 level in the liver may be up-regulated by SFA or HFD through SREBPs activation. PPARs are also well-known transcription factors activated by HFD, and are strongly implicated in T2DM and metabolic syndrome [31]. Another adipogenic transcription factor, CEBPA is activated by high energy nutrients [32]. Furthermore, the levels of CEBPA and p53 expression are up-regulated in DIO mice [32,33]. Although further study will be needed, the up-regulation of miR-195 in hepatocytes might be triggered through the activation of certain transcription factors regulated by SFA or DIO.

This study showed that the ectopic expression of miR-195 suppresses the mRNA and protein of INSR, but not IRS-1, in hepatocytes (Fig. 4). In addition, the ectopic accumulation of SFA causes a substantial decrease in IRS-1 and INSR expression, both at the transcriptional and post-transcriptional levels [1,7]. In this process, SFA reduces the IRS-1 level transcriptionally and post-transcriptionally [1,7]. Although *in silico* analysis suggested IRS-1 to be a target of miR-195, the results from the Dual luciferase assay and ectopic expression clearly suggest that IRS-1 is not a direct target gene of miR-195 in human hepatocytes. Therefore, miR-195 is considered a causative component in INSR suppression in hepatocytes, except for IRS-1, at the post-transcriptional level. Recently, Mortuza *et al.* reported that miR-195 expression was up-regulated in the retinas of diabetic rats, leading to down-regulation of Sirt1 [34]. Sirt1 is a protein deacetylase that has been implicated in the regulation of metabolic activity, and its expression is decreased in insulin resistance, oxidative stress, and inflammation [35]. If Sirt1, in consort with AMPK, is a component of the adaptive response against insulin resistance and a dysregulation of the metabolic activity, miR-195 may aggravate insulin resistance, T2DM, and metabolic syndrome.

In conclusion, miR-195 is induced by SFA palmitate, and suppresses INSR, but not IRS-1, by targeting *INSR* 3'UTR directly. The expression of miR-195 is also up-regulated substantially in the liver of DIO mice. The ectopic expression of miR-195 results in a drastic repression of INSR protein expression, and eventually causes impaired insulin signaling and glycogen synthesis in HepG2 cells. Overall, these results clearly suggest that miR-195 is causally involved in the development of insulin resistance induced by SFA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2014.09.006>.

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